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The Effects of Exogenous Cytokinin on the Morphology and Gender Expression of *Osmunda regalis* Gametophytes

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ABSTRACT.—The goal of this study was to determine the function of cytokinin in the morphological development and gender expression of gametophytes of *Osmunda regalis*, a member of Osmundales, sister-group to all other extant leptoporangiate fern families. Gametophytes of *Osmunda regalis* were grown in multispore populations on C-fern nutrient enriched agar containing 0, 1 nM, 1 μM, and 1 mM kinetin. Higher concentrations of exogenous kinetin reduced gametophyte size (area), disrupted correlations observed in the control between rates of apical notch formation and thallus widening, increased the proportions of asexuals and males, decreased the proportion of females in the population, and correspondingly increased male reproductive effort (antheridia per unit thallus area) and decreased female reproductive effort (archegonia per unit thallus area) compared to the control. Low concentrations of exogenous kinetin increased the proportion of females compared to the control. In the control, thalli with a comparatively deep apical notch tended to be wider (relative to their length) and possess a more circular silhouette relative to thalli with a comparatively shallow apical notch; however, these morphological parameters were independent of gametophyte size and gender. Thus, variance in the rate and planes of cell division and patterns of cell expansion and differentiation, most likely genetic in basis, were observed in the control, and the observed effects of exogenous kinetin were more than a simple "push" towards a phenotype already present in the control.

KEY WORDS.—cytokinin, development, kinetin, leptosporangiate, fern, gametophyte, morphology, *Osmunda regalis*, gender expression

Current models of phytohormonal control of seed plant shoot apical meristems (SAM) emphasize the role of cytokinin (Stahl and Simon, 2010; Kurakawa *et al.*, 2007; Kyozuka, 2007; Sablowski, 2007; Hwang and Sakakibara 2006; Kepinski, 2006; Shani *et al.*, 2006; Hudson, 2005; Rashotte *et al.*, 2005; Higuchi *et al.*, 2004). Cytokinin maintains indeterminism and stimulates cell division in SAM and apical dominance and other source sink relationships. Cytokinins are also involved in a wide range of developmental processes including vascular development and senescence (Mok and Mok, 2001), phyllotaxy (Guilini *et al.*, 2004), cotyledon expansion (Stoynova-Bakalova *et al.*, 2003; Huff and Ross, 1975), and chloroplast maturation (Stetler and Laetsch, 1965), and they are involved in a wide range of responses to abiotic and biotic stimuli, including light responses, drought resistance, ion uptake, pathogen defense and symbiont interaction (Werner and Schmülling, 2009). In contrast, cytokinins have the opposite developmental role in the root apical

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meristem, where they control root meristem size via regulation of auxin distribution (which stimulates cell proliferation and maintains totipotency in the root apical meristem (RAM); Stahl and Simon, 2010; Růžička *et al.*, 2009), stimulate elongation and differentiation in the transition zone (Moubayidin *et al.*, 2009; Ioio *et al.*, 2008; Kyozuka, 2007), and stimulate root nodulation (Frugier *et al.*, 2008). Plant reproduction is also governed by SAM behavior; however, mechanisms governing gender expression and reproductive effort (unit investment into reproduction per unit vegetative investment) vary widely among and within plant groups (Tanurdzic and Banks, 2004). In taxa where phytohormones rather than sex-determining genes control gender expression, cytokinins are associated with reproduction (e.g., flowering) and femaleness (Tanurdzic and Banks, 2004; Khryanin, 2002; Lejeune *et al.*, 1988).

In mosses, cytokinins also play a key role in SAM behavior in the gametophyte, governing the transition from a filamentous protonema to a 3-dimensional leafy thallus (Cove *et al.*, 2006). Whereas picomolar concentrations of kinetin initiate the formation of caulonema initials that produce filamentous growth, micromolar concentrations stimulate the assembly of 3-dimensional buds in *Funaria hygrometrica* Hedwig (Schumaker and Dietrich, 1997; Bopp and Jacob, 1986). This change in morphology was initiated by a dramatic swelling of the initial and a subsequent change from periclinal to oblique and anticlinal divisions. Our search of the literature failed to find studies investigating the role of cytokinins in moss reproduction.

Among the majority of leptosporangiate families, morphological development of the gametophyte proceeds from spore germination through filamentous and spathulate forms culminating in a cordate form that exhibits taxon-specific propensities for subsequent elongation and branching (Nayar and Kaur, 1971). The transition from a spathulate to a cordate form reflects an increase in the rate of anticlinal divisions (i.e., perpendicular to the apical surface), as opposed to periclinal and oblique divisions, in a single plane within the single-celled or pluricellular apical meristem and prolonged meristematic activity by its derivatives (von Aderkas and Cutter, 1983). Formation of the apical notch characteristic of a cordate morphology is reproductively significant in the taxa in which it occurs as it always precedes female gender expression (i.e., production of archegonia). In species with an antheridiogen system, sensitivity to antheridiogen diminishes with the onset of an apical notch. Thus, to the extent that cytokinin influences the rate and orientation of cell division of the apical meristem, it governs morphological form and gender expression in leptosporangiate fern gametophytes.

Cytokinins have been identified in leptosporangiate fern sporophytes (Stirk and van Staden, 2003 and citations therein); however, our search of the literature did not any find reports regarding cytokinin functions in these plants. Similarly, we found only two reports regarding the role of cytokinins in leptosporangiate gametophyte development (Menendez *et al.*, 2009; Spiro *et al.*, 2004). Pico and nanomolar concentrations of kinetin (BAP, 6-Benzylaminopurine) induced formation of the notch meristem *Ceratopteris richardii* Brongn. grown in the dark, resulting in a decrease in thallus length:

width ratio (Spiro *et al.*, 2004), partially overcoming etiolation. Exogenous cytokinin did not accelerate the rate of reproductive maturation in this study; however, the possibility of a delay in reproductive maturity or effects on gender expression and reproductive effort (gametangia per unit thallus area) were not explored. In contrast, micromolar concentrations of exogenous BAP delayed formation of an apical notch and production of gametangia in light-grown gametophytes of *Blechnum spicant* (L.) Smith (Menendez *et al.*, 2009). In the same study, endogenous levels of six cytokinins were found to be higher in female than in male gametophytes in this species. These observations establish the presence of cytokinins in sporophytes and gametophytes of leptosporangiate ferns, the influence of light on cytokinin response by gametophytes, and that, like seed plants, cytokinins are associated with the maintenance of an apical meristem and female gender expression of gametophytes.

The study we report here investigated the effects of exogenous kinetin on morphological development, gender expression, and reproductive effort in *Osmunda regalis* L., a member of Osmundales, sister-group to all other extant leptosporangiate ferns (Smith *et al.*, 2006; Pryer *et al.*, 2004). Our goal was to generate information regarding cytokinin that may be useful in reconstructing the evolution of developmental mechanisms in leptosporangiate fern gametophytes.

Materials and Methods

Spores of the fern *O. regalis* were collected from a minimum of three sporophytes growing in Pigeon Creek Park, Ottawa County, Michigan, and stored in aggregate for 10 days at 3°C. Preliminary experiments using spore sterilization and antibiologicals (Nystatin® and streptomycin) resulted in reduced germination and atypical morphology, namely branched rather than a non-branched, globular form. Thus, the green spores of this species were repeatedly centrifugally rinsed, but, not surface sterilized in our study. No bacterial, fungal, or algal contaminations were observed throughout the study.

Concentrations (1 nM, 1 μM, and 1 mM) of kinetin were prepared with C-fern nutrient media (Carolina Biological Supply) and 1.5% agar with the control group containing only nutrient media. Each treatment was replicated five times. Spores were sown at an approximate density of four per cm^2 and the plates arranged in a fully randomized design under full spectrum lights producing light levels of approximately 29.5 μmol^{-1}, m^{-2}, sec^{-1} at dish level using a 16 hour light: 8 hour dark regime at 20°C.

Data collection.—Six weeks after sowing, gametophytes were harvested haphazardly from the lowest density neighborhoods within each plate, totaling 30–35 gametophytes per treatment. Although efforts were made to create an even density of gametophytes within each plate, variation occurred. Gametophytes in high-density neighborhoods are subject to competitive interactions resulting in reduced growth rate (Greer, 1993; Huang *et al.*, 2004) and more vulnerable to damage during harvest and were therefore avoided. The harvested gametophytes were fixed with Clarion© mounting

medium as semi-permanent slides. Dissecting microscopes were used under uniform magnification to digitally photograph each gametophyte. Morphological traits were measured in pixels using the trace measurements feature in SigmaScan Pro 5.0 (Fox and Urich, 1993) following the methodology of Greer and Curry (2004) and included thallus area (size), thallus length, thallus width and notch depth. Thallus length extends from the caudal "tail" to the apical notch. Notch depth was measured as an extension of the thallus length line terminated by a line that connects the anterior apex of each thallus lobe. Thallus width was measured as the longest possible line perpendicular to the thallus length line. Silhouettes are shown at the same scale; scale shown with silhouette taken from 1 nM kinetin treatment. Three morphological metrics, thallus length / width ratio (LW), notch depth / thallus length ratio (NDL), and shape factor (SF = circularity = $4\pi \times$ (object area / perimeter^2); 0 = line, 1.0 = a perfect circle) were used to assess developmental status, namely development of an apical notch. As a fern gametophyte develops, its NDL and SF are expected to increase and its LW decrease as a notch-bearing apical meristem develops and, subsequently, widening growth outpaces lengthening growth. Although included here for comparative purposes, Greer *et al.* (2009) rejected shape factor as a reliable metric of developmental status. Each gametophyte was also scored for the number of antheridia and archegonia present.

Data analyses.—One-way parametric or non-parametric (Kruskal-Wallis) ANOVA were used to test for treatment effects on thallus size and shape. Bonferroni multiple comparisons were used following significant parametric ANOVA and Tamhane's T2 multiple comparisons were used following significant Kruskal-Wallis ANOVA. Spearman's ranked correlations were used to compare relationships among thallus size and the three morphological metrics within each kinetin treatment group.

Chi-square tests of independence including all four kinetin concentrations were used to test for treatment effects on gender ratios. When a multi-treatment chi-square was found significant, pair-wise chi-square tests of independence were performed between all six treatments pairings. Parametric and Kruskal-Wallis ANOVA were used to test for treatment effects on numbers of antheridia and archegonia per gametophyte after adjusting for thallus size and each of the three morphological metrics; i.e., size and morphologically-based measures of reproductive effort. Bonferroni and Tamhane's multiple comparisons were used accordingly.

Assumptions of normality and homogeneity were tested using Kolmogorov-Smirnov's and Levene's tests respectively. P-values were considered significant when ≤ 0.05 and marginally significant when between 0.05 and 0.10. When warranted, P-values were adjusted using Bonferroni-Holm's correction for multiple comparison error rates.

Results

Treatment effects on morphology.—All gametophytes were cordate at harvest. Of the morphological traits assessed, only gametophyte area differed

among kinetin treatments (Table 1). Gametophytes exposed to the highest kinetin concentration (1 mM) were approximately 50% smaller than gametophytes all other treatment groups (Fig. 1a, e–h). Visually consistent but statistically non-significant declines in LW and SF, and to a lesser extent NDL, were observed with increasing kinetin concentration (Fig. 1b–h).

Thallus area did not correlate with the three morphological metrics within any treatment group. Among the three morphological metrics, correlations involving NDL were the strongest and most frequently significant, whereas correlations involving SF were the weakest and least frequently significant (Table 2). Thallus NDL correlated negatively with LW in all treatments (maximum $R^2 = -0.404$ in the 1nM Kinetin treatment) except within the highest (1 mM) kinetin treatment. Similarly, NDL correlated negatively with SF in the control and lowest (1 nM) kinetin treatment (Table 2). LW correlated positively with SF (maximum $R^2 = 0.258$) only in the 1 μM kinetin treatment. Thus, control thalli possessing deeply formed apical notches tended to have wider thallus widths relative to their lengths and more linear silhouettes than thalli with shallowly formed apical notches (Fig. 2), and these relationships weakened (i.e., became non-significant) at higher (1 μM and 1 mM) kinetin concentrations (Fig. 1).

Of the three metrics used to assess the morphology of *O. regalis* gametophytes, NDL correlated more strongly with LW and SF, than either of the latter with one another. A similar observation was made by Greer *et al.* (2009) in assessing gametophyte morphology of *O. regalis* and *Athyrium filix-femina* (L.) Mertens, thus, NDL appears to be the most reliable of these metrics for assessing the developmental status of leptosporangiate species with gametophytes that form an apical-notch. However, each metric provides a slightly different insight and we recommend using all three metrics when assessing gametophyte morphology and reproductive effort.

TABLE 1. ANOVA of morphological traits of *Osmunda regalis* gametophytes exposed to 0, 1 nM, 1 μM, and 1 mM concentrations of kinetin.

Parametric ANOVA	Groups	Df	MS	F	P
Thallus Area	Between	3	5.31×10^{11}	40.04	<0.001
	Within	128	1.32×10^{10}		
	Total	131			
Thallus Length / Width (LW)	Between	3	0.006	1.88	0.135
	Within	128	0.003		
	Total	131			
Notch depth / Thallus Length (NDL)	Between	3	0.001	0.039	0.990
	Within	128	0.012		
	Total	131			
Thallus Shape Factor (SF)	Between	3	0.006	1.32	0.271
	Within	128	0.004		
	Total	131			

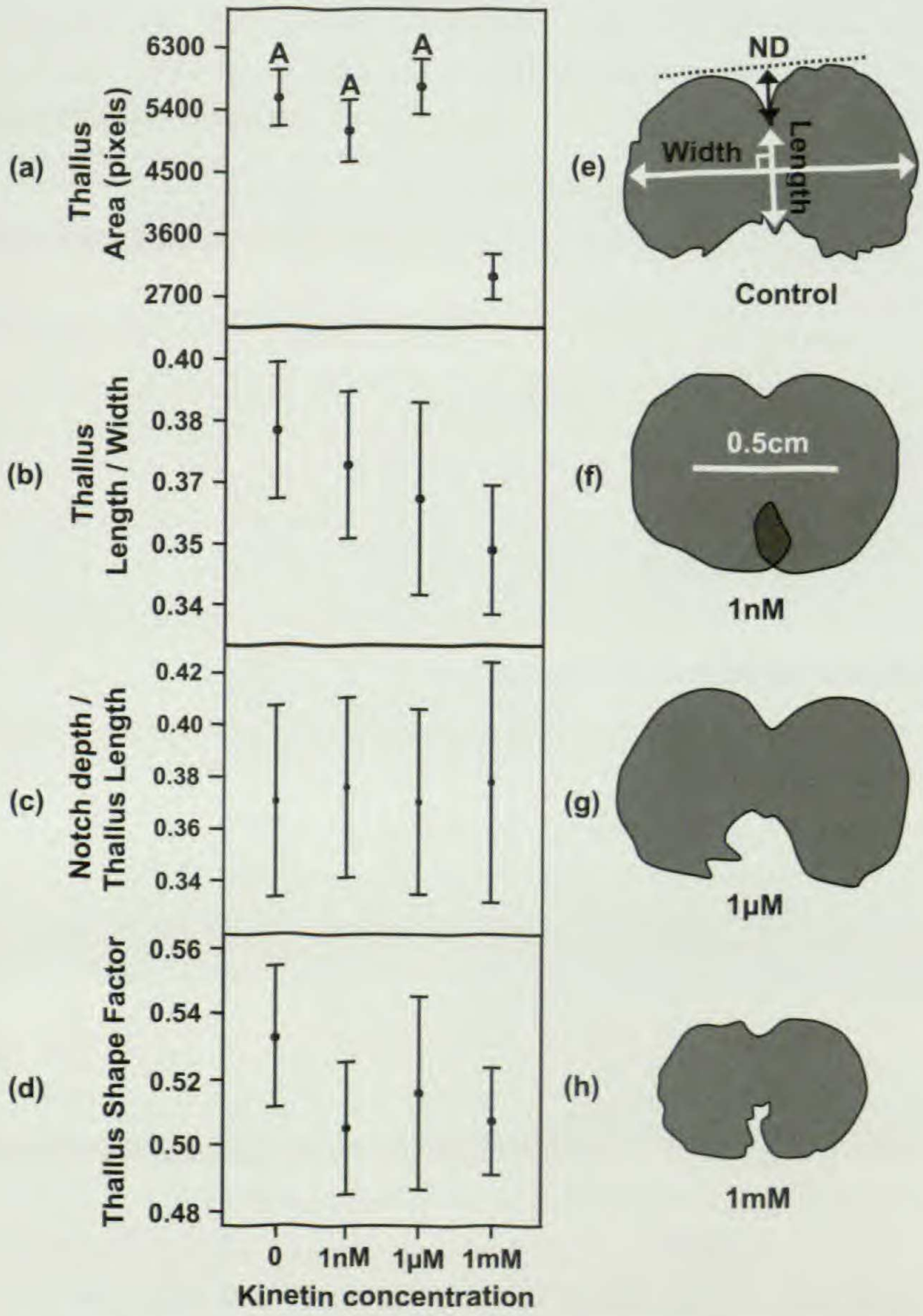

FIG. 1. Morphological measurements (a–d) and representative silhouettes (e–i) of *O. regalis* gametophytes measured in pixels in response to 0 (control), 1 nM, 1 μM, and 1 mM kinetin treatments. Treatments with the same letter are not significantly different ($P > 0.05$) from one another based on Bonferroni multiple comparison tests (a–d). Linear morphological measurements are shown with the silhouette taken from the control and are described in the methods.

Treatment effects on reproductive traits.—No differences in thallus area, NDL, LW or SF were observed among gender categories within the control or 1 mM kinetin treatments (Table 3).

Gender ratios differed only between the highest (1 mM) kinetin treatment group and all lower treatment groups with one exception (Fig. 3). The proportion of asexuals and males in the 1 mM kinetin treatment group increased significantly relative to the control by 48% and 876% (0.48 and 8.76-fold) increase, respectively (Fig. 3). Expected values for males in the lower kinetin treatment groups fell below five therefore results from chi-square tests for this gender should be viewed with caution. Correspondingly, the

TABLE 2. Spearman's ranked correlation coefficients (R^2) between morphological traits among *Osmunda regalis* gametophytes exposed to 0 (control), 1 nm, 1 μM and 1 mM kinetin treatments. Asterisks indicate P-value ≤ 0.05. N = number of gametophytes per treatment group.

	Thallus Length / Width (LW)	Notch Depth / Thallus Length (NDL)	Thallus Shape Factor (SF)
Control (N = 42)			
Area	−0.022	−0.021	−0.0003
LW		−0.320*	0.058
NDL			−0.149*
1 nM Kinetin (N = 26)			
Area	−0.011	0.027	−0.032
LW		−0.404*	0.258*
NDL			−0.309*
1 μM Kinetin (N = 32)			
Area	−0.230*	−0.003	−0.016
LW		−0.325*	0.001
NDL			−0.076
1 mM Kinetin (N = 32)			
Area	−0.0007	−0.041	0.005
LW		0.047	0.082
NDL			−0.062

proportions of females in the 1 mM kinetin treatment group decreased significantly (58%) relative to the control (Fig. 3). The only other kinetin treatment with detectable effects on gender expression was the lowest (1 nm) kinetin treatment, where the proportion of females was marginally ($P < 1.0$) greater than in the control or 1 mM kinetin treatment (Fig. 3). Differences in the proportion of hermaphrodites were non-significant among all treatment groups.

Significant differences in the number of antheridia per antheridium-bearing gametophyte were detected only when corrected for thallus area (Table 4, Fig. 4a–d). Gametophytes in the 1 mM treatment groups possessed more antheridia after correcting for size than in all lower kinetin treatment groups (Fig. 4a). Similarly, gametophytes in the second highest (1 μM) kinetin treatment group possessed more antheridia than those in the control (Fig. 4a).

In contrast, no significant differences in the number of archegonia per archegonium-bearing gametophyte were observed when corrected for thallus area, but were observed when corrected for each of the three morphological metrics (Table 5, Fig. 4e–h). Gametophytes in the 1mM treatment group possessed fewer archegonia, when corrected for NDL, LW or SF, than those in the control and lower kinetin treatments (Table 5, Fig. 4e–h). Interpretation of gametophyte reproductive effort is straightforward when gametangium production is adjusted for thallus area; however, adjustments using morphological metrics that are themselves fractions (e.g., number of archegonia / (notch depth / thallus length)) require careful interpretation as there are three means by which such a measure of reproductive effort can change in the same

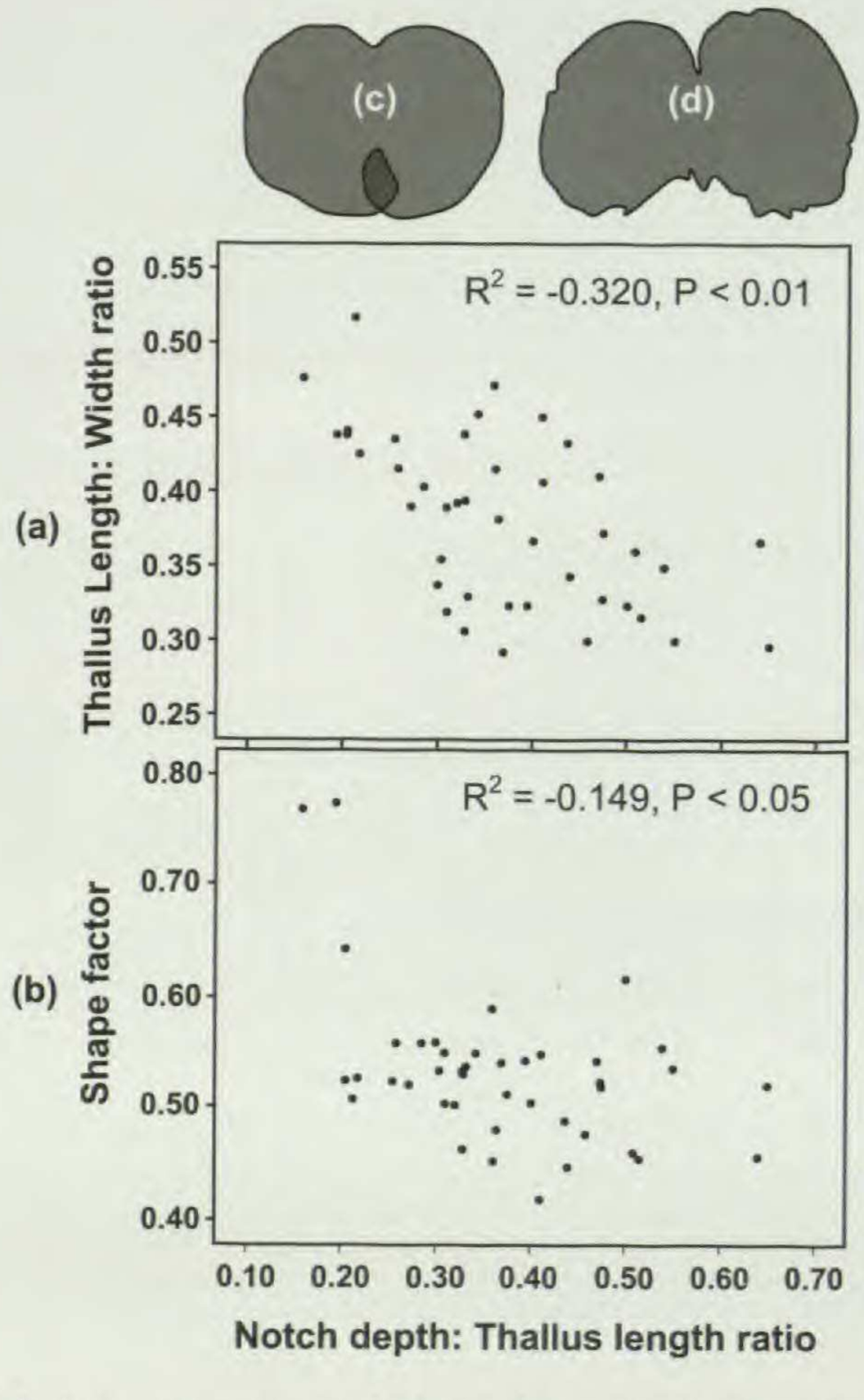

FIG. 2. Significant correlations among morphological metrics within the control. Scatterplots of notch depth: thallus length ratio versus (a) thallus length: width ratio and (b) shape factor. R^2 and P-values taken from Table 2. Silhouettes of representative thalli possessing comparatively low NDL and high LW and SF (c) and comparatively high NDL and low LW and SF (d); shown at same scale.

direction. Three comparisons between the morphological and reproductive data support the conclusion that the decline in the 1 mM kinetin treatment in archegonium production when corrected for morphological status reflects a biologically meaningful decrease in reproductive effort: (1) thallus LW declined (visually) in stepwise manner (Fig. 1b), whereas archegonia / LW remained essentially constant among the lower kinetin treatments and declined 51% in the 1 mM kinetin treatment relative to the control (Fig. 4b); (2) NDL showed no visual change with increasing kinetin concentration (Fig. 1c) yet archegonia / NDL declined 46% in the 1 mM treatment relative to the control (Fig. 4c); (3) SF decline began (visually) with the 1 nM kinetin treatment (Fig. 1d), whereas archegonia / SF declined 53%, relative to the control, only at 1mM kinetin treatment (Fig. 4d).

DISCUSSION

At the higher concentrations used in this study (1 μM and 1 mM), exogenous kinetin reduced the size (area) of *O. regalis* gametophytes, disrupted the

TABLE 3. Kruskal-Wallis ANOVA of thallus size and morphology among gender categories in the control and 1 mM kinetin treatments. Only one male occurred within the control; however, exclusion of males did not change the outcome of the analysis and were therefore retained here. NDL = Notch depth / Thallus length. LW = Thallus length / width. SF = Shape factor. N = number of gametophytes per treatment group.

		Control				1 mM Kinetin			
Trait	Gender	N	Mean rank	χ^2	P	N	Mean rank	χ^2	P
Area	Asexual	8	19.25	3.506	0.320	9	14.44	0.742	0.863
	Male*	1	21.00			7	16.29		
	Female	22	20.18			7	18.14		
	Hermaphrodite	11	24.00			9	17.44		
NDL	Asexual	8	22.00	2.037	0.565	9	13.67	0.714	0.870
	Male*	1	15.00			7	16.43		
	Female	22	21.23			7	19.86		
	Hermaphrodite	11	22.27			9	16.78		
LW	Asexual	8	17.88	1.005	0.800	9	14.67	0.793	0.793
	Male*	1	24.00			7	17.00		
	Female	22	21.77			7	19.29		
	Hermaphrodite	11	23.36			9	15.78		
SF	Asexual	8	21.63	0.349	0.951	9	18.22	1.726	0.631
	Male*	1	11.00			7	17.00		
	Female	22	23.50			7	16.29		
	Hermaphrodite	11	18.34			9	14.56		

*Exclusion of males did not change the significance of these analyses and were therefore retained.

positive correlation between apical notch formation (NDL) and the rate of thallus widening (LW and SF) observed in the control, increased the proportions of asexuals and males and decreased the proportion of females in the population, and correspondingly increased male reproductive effort and decreased female reproductive effort. In the control, thalli with a comparatively deep apical notch tended to be wider (relative to length) and possess a more circular silhouette relative to thalli with a comparatively shallow apical notch; however, morphological status was independent of gametophyte size. These observations reveal variance, potentially genetic in basis, in the prevailing rate and planes of cell division and patterns of cell expansion and differentiation. Variations in gametophyte size and morphology in the control were not associated with differences in gender, therefore the effects of exogenous kinetin treatment on notch development, thallus widening, gender expression, and reproductive effort were more than a simple "push" towards a phenotype already present in the control. In contrast, Huang *et al.* (2004) observed the following size hierarchy among one year-old gametophytes of *Osmundastrum cinnamomeum* (L.) C. Presl: female – hermaphrodite – male – asexual, with females three times larger than hermaphrodites. *Osmundastrum* is sister to all other genera within extant Osmundales (Metzgar *et al.*, 2008). Assuming *O. regalis* exhibits similar gender-based size hierarchies at the same age and densities as *Osmundastrum*, latent differences in size or, correspondingly,

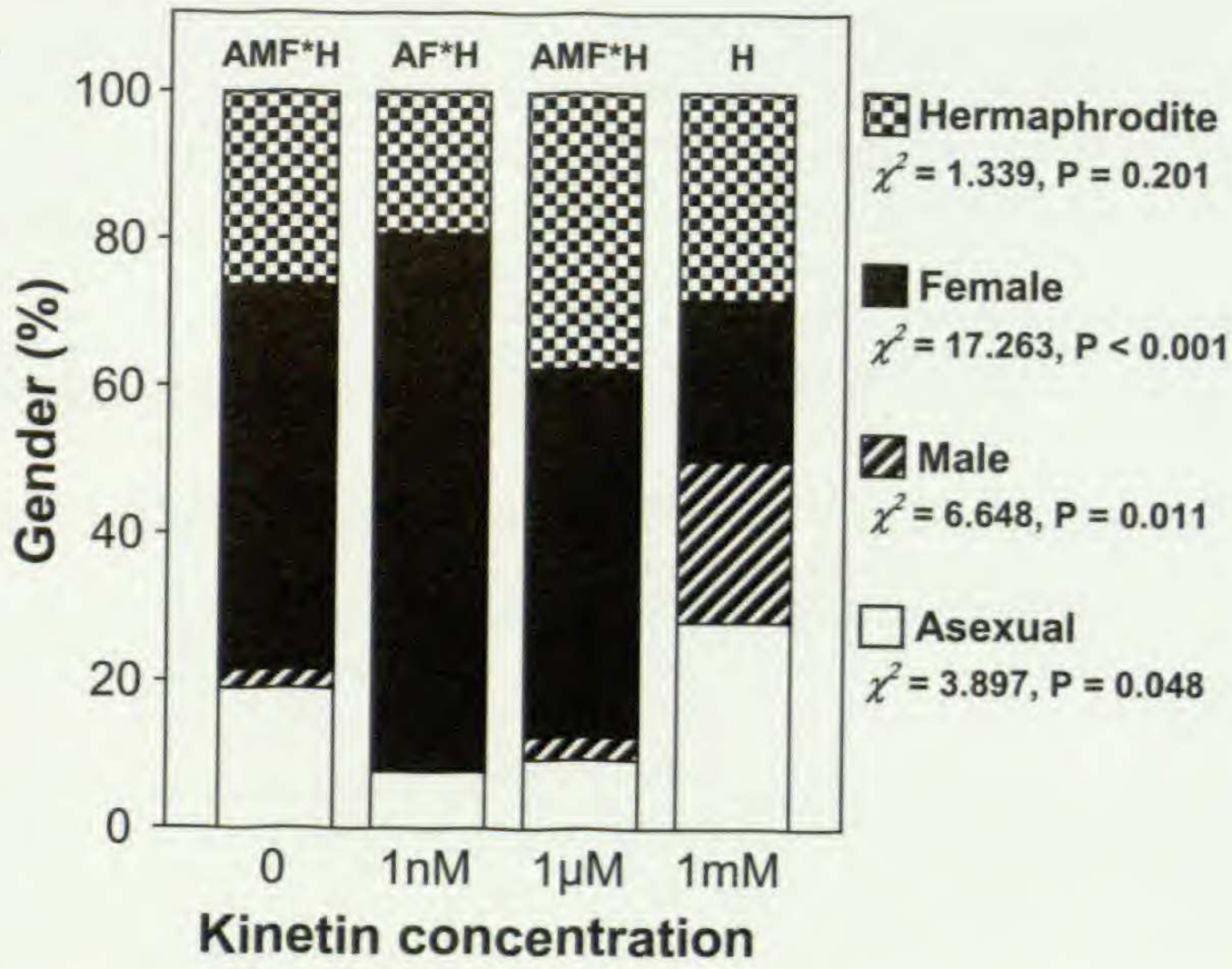

FIG. 3. Gender ratios in multispore populations of *O. regalis* exposed to 0 (control), 1 nM, 1 μM, and 1 mM kinetin treatments. Results for chi-square tests of independence for each gender across all kinetin treatments are listed below each legend. Treatments that possess the same letter are not significantly different from one another ($P > 0.05$) based on pair-wise chi-square tests of independence following Bonferroni-Holm's correction for multiple comparison error rates; however, the proportion of female gametophytes was marginally greater ($P < 0.10$) in the 1 nM kinetin treatment than in the control and 1 μM kinetin treatment, as indicated by the asterisk.

growth rate in six week-old gametophytes have on gender expression increase with gametophyte age.

Production of archegonia in *O. regalis*, as with all known cordate-forming leptosporangiate ferns, is preceded by the formation of a pluricellular apical meristem and corresponding apical notch. Reduction of female reproductive effort by exogenous kinetin was largely independent of thallus area and closely associated with measures of cordate morphology (i.e., NDL, LW and SF), reflecting the activity of the apical meristem.

In contrast, the stimulating effect of high levels of exogenous kinetin on the frequency of males and on male reproductive effort (i.e., the rate of antheridium production) was associated with its reducing effect on thallus area. All gametophytes in this experiment were observed only once, six-weeks after spore sowing, and all were cordate, therefore the timing of antheridium development relative to the development of an apical notch is unknown. The sequence of gender expression in Osmundales is poorly known. A male to hermaphrodite sequence was reported for *Osmundastrum cinnamomea* (Huang *et al.*, 2004) which, as noted above is sister to all other extant genera within Osmundales, and *O. regalis* (Klekowski, 1973), and *Todea barbara* (L.) T. Moore (von Aderkas and Cutter, 1983); however, the relationship between apical notch formation and antheridium production was not reported in these studies. In a time-series study of multispore populations of congeners *O. lancea* Thunb. and *O. japonica* Houtt., Hiyama *et al.* (1992) observed antheridia only after, or corresponding to, the formation of a cordate

TABLE 4. ANOVA of morphologically adjusted rates of antheridium production among male and hermaphrodite *O. regalis* gametophytes exposed to 0 (control), 1 nM, 1 μM and 1 mM concentrations of kinetin. NDL = Notch depth / Thallus length. LW = Thallus length / width. SF = Shape factor. N = number of gametophytes per treatment group.

Parametric ANOVA	Groups	df	MS	F	P
Antheridia / NDL	Between	3	4.917	0.306	0.821
	Within	42			
	Total	45			
Kruskal-Wallace ANOVA	**Kinetin Concentration**	**N**	**Mean Rank**	**χ^2**	**P**
Antheridia / Area	0	12	14.75	15.56	0.002
	1 nM	5	17.20		
	1 μM	13	22.15		
	1 mM	16	33.13		
Antheridia / LW	0	12	18.17	2.856	0.414
	1 nM	5	23.80		
	1 μM	13	24.38		
	1 mM	16	26.69		
Antheridia / SF	0	12	20.00	1.380	0.710
	1 nM	5	22.80		
	1 μM	13	26.15		
	1 mM	16	24.19		

morphology. Similarly antheridia were observed only after the formation of an apical notch in a time-series of *O. cinnamomeum* isolates, (Hollingsworth *et al.*, in press). Thus, antheridium production in *Osmundastrum* and at least two species of *Osmunda* appears to be dependent upon the formation of an apical notch. If cordate dependence of antheridium production exists in *O. regalis* as well, it would explain why the effect of exogenous kinetin on antheridium production was independent of NDL, LW or SF. The rate of antheridium production in *O. regalis* also appears to be largely independent of NDL, LW and SF among cordate gametophytes, but is dependent on area as evidenced in the control. This may reflect the fact that antheridia were produced in the basal region, the region most independent of the influence of the apical meristem. As was observed in the control, variations in size and morphology were not associated with differences in gender in the highest (1 μM) kinetin treatment. Thus, the effects of kinetin on female and male reproductive efforts appear to be independent of latent, potentially genetic, differences in the rates of growth and development. The absence of males in the lowest kinetin treatment (1 nM) group and corresponding increase in the proportion of females relative to the control and 1 μM kinetin treatment supports the hypothesis that cytokinin effects on gender expression are concentration dependent.

The morphological and reproductive changes in *O. regalis* induced by high levels of exogenous kinetin are most readily explained by one or more of the following effects on the apical meristem: (1) an increase in the ratio of anticlinal versus periclinal-oblique divisions, (2) reduced expansion of derivatives, and (3) a delay in the differentiation of the derivatives.

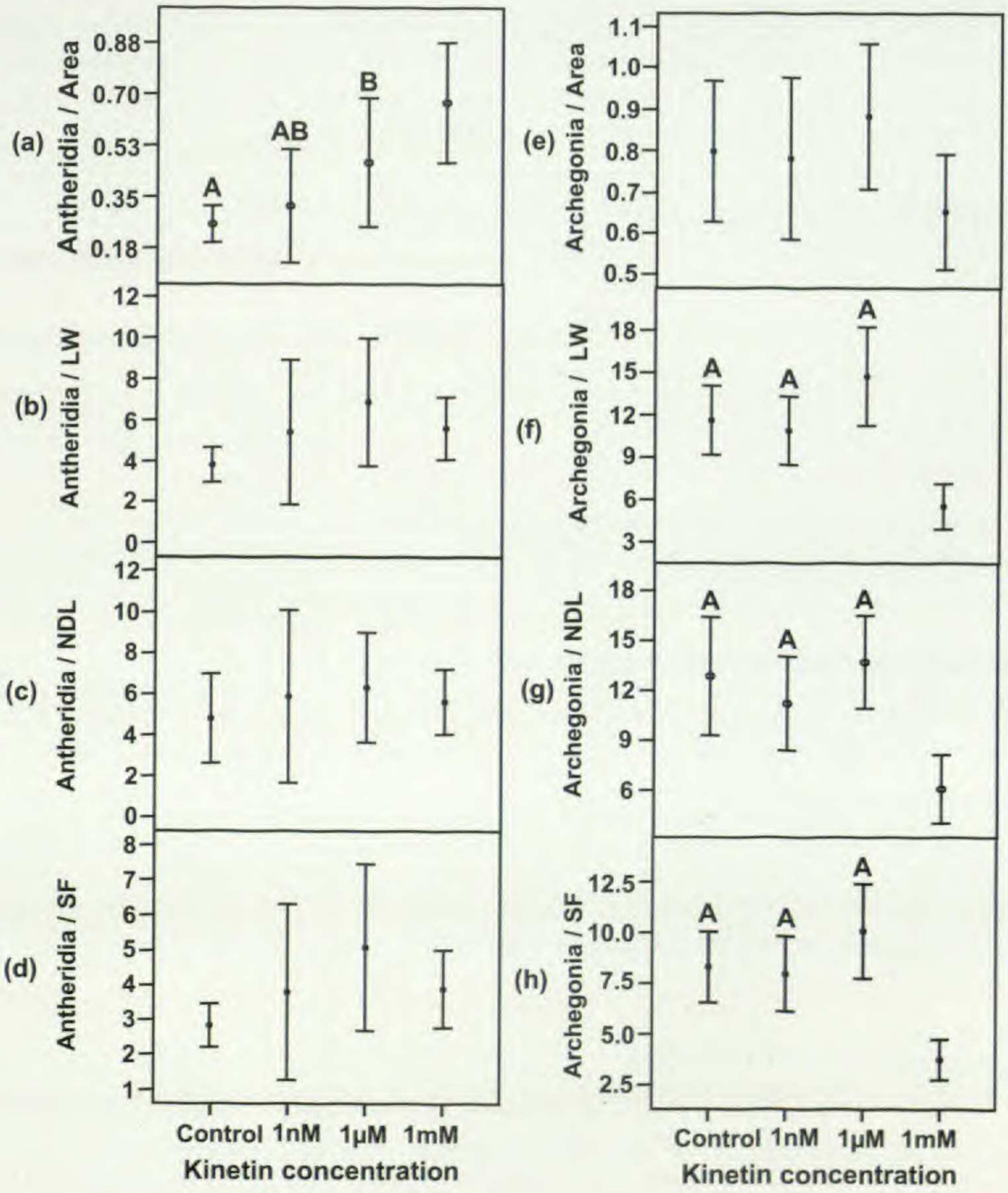

FIG. 4. Production of gametangia per *O. regalis* gametophyte after correcting for size (area) and morphology in response to 0 (control), 1 nM, 1 µM, and 1 mM kinetin treatments. Archegonia per pixel area was multiplied 100 fold for visual purposes. Treatments that possess the same letter are not significantly different ($P > 0.05$) from one another based on Bonferroni and Tamhane's T2 multiple-comparisons tests.

Greer *et al.* (2009) also increased the cytokinin: gibberellin ratio in *O. regalis;* however, they did so by lowering endogenous gibberellin levels. Nevertheless, they also observed an increase in the proportion of asexual and male gametophytes, suggesting that the cytokinin: gibberellin ratio may be more important than absolute phytohormone levels in determining the specifics of cell division and differentiation. If a high cytokinin: gibberellin ratio is necessary for production of archegonia, then these observations emphasize that exogenous cytokinins do not precisely mimic the effects of endogenous cytokinins as revealed by the control in the present study.

In contrast to the present study using kinetin, exogenous cytokinin (BAP) accelerated the development of an apical notch and significantly decreased

TABLE 5. ANOVA of morphologically adjusted rates of archegonium production among female and hermaphrodite *O. regalis* gametophytes exposed to 0 (control), 1 nM, 1 μM, and 1 mM concentrations of kinetin. NDL = Notch depth / Thallus length. LW = Thallus length / width. SF = Shape factor. N = number of gametophytes per treatment group.

Parametric ANOVA	Groups	df	MS	F	P
Archegonia / Thallus Area	Between	3	0.185	0.891	0.449
	Within	97			
	Total	100			
Kruskal-Wallace ANOVA	**Kinetin Concentration**	**N**	**Mean Rank**	**χ^2**	**P**
Archegonia / LW	0	33	52.15	14.52	0.002
	1 nM	24	52.33		
	1 μM	28	62.04		
	1 mM	16	27.31		
Archegonia / NDL	0	33	69.57	26.24	<0.001
	1 nM	24	77.08		
	1 μM	28	82.56		
	1 mM	16	37.81		
Archegonia / SF	0	33	53.64	15.71	0.001
	1 nM	24	53.13		
	1 μM	28	60.71		
	1 mM	16	25.38		

LW in dark-grown gametophytes of *Ceratopteris richardii* (Spiro *et al.*, 2004). Similarly, exogenous BAP reduced LW and delayed the production of both antheridia and archegonia in *Blechnum spicant* and endogenous levels of six cytokinins were higher in females than in males (Menendez *et al.*, 2009). The different results between these studies of core-leptosporangiate species and the present study of a member of the Osmundales may underscore phylogenetically relevant differences in phytohormonal controls of development.

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